CONTRIBUTIONS TO THE MICROBIOLOGY OF AUSTRALIAN SOILS. III.

THE ROSSI-CHOLODNY METHOD AS A QUANTITATIVE INDEX OF THE GROWTH OF FUNGI IN THE SOIL, WITH SOME PRELIMINARY OBSERVATIONS ON THE INFLUENCE OF ORGANIC MATTER ON THE SOIL MICROFLORA.

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(Two Text-figures.)

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Introduction.

Although a great deal of research work has already been carried out on the influence of various organic compounds upon the abundance and the composition of the soil microflora, we have yet very little information concerning the combined influence of organic matter and varying temperatures and moisture-degrees on the general composition of the soil microflora, in spite of the profound influence which these factors are known to exert upon the course of the decomposition of organic matter in the soil. The results of a number of preliminary experiments in this direction are presented in this contribution.

To a number of samples of soils of varying character were added various kinds of organic material, usually one per cent. on the basis of air-dry soil, whereupon the soils were adjusted to the desired degree of moisture and incubated at different temperatures for periods up to 15 days. The numbers of bacteria, actinomycetes and fungi were determined by plate counting, and in addition the development of fungal mycelium was controlled on microscopic slides placed in the soil.* The arrangement of the experiments as well as the methods of counting and of staining of the slides were essentially the same as previously described (Jensen, 1934*b*), except that somewhat smaller quantities of soil were generally used, and when a slide was removed from the soil, a new one was placed instead of it (cf. Conn, 1932). The agar plates were incubated at 27–28°C. At an early stage of the work it was realized that the semi-quantitative description of the slides as more or less "rich" or "poor" in fungal mycelium is unsatisfactory. A more precise estimate of the density of mycelium was therefore sought by examining a large number of microscopic fields (usually 500–550), distributed as evenly as possible over the slide, and calculating the percentage of fields showing the presence of fungal hyphae. An oil immersion objective (Leitz 1/12, n. ap. 1.30) and a low-power eyepiece were used, and only a central square field of approximately 65μ side-length was examined. This method, while not enabling us to express the quantity of mycelium in terms of the weight of soil, gives a good picture of the richness of different soils in fungal mycelium. At the same time the numbers of fungal spores, where present,

* The method introduced by G. Rossi and N. Cholodny (Jensen, 1934*b*).

were counted. These counts, however, cannot be taken as reliable indices of the actual content of spores; since most fungi produce their spores in clusters or chains, the distribution of the spores over the slides is not random, and the counts do not follow the Poisson series (Fisher, 1930). Moreover, it is not always easy to distinguish microscopically between solitary fungal spores and encysted protozoa, especially flagellates. Counting of the bacteria and actinomycetes on the slides was found impossible because of the frequent occurrence of fields too densely crowded with organisms to admit of any counting.

Experimental Results.

The results of this series of experiments are reproduced in Table 1. Several interesting facts emerge from these figures. Firstly, the numbers of bacteria show in parallel experiments almost constantly a decrease with increasing temperature, after 4–5 as well as after 12–14 days. An increase in the moisture is frequently, but by no means constantly, accompanied by an increase in the bacterial numbers. The numbers of actinomycetes, on the other hand, show in the large majority of cases a definite increase with increasing temperature, although there is little difference in their numbers at 27–28° and at 37–40° C. Increases in the moisture seem to tend to depress rather than to increase their numbers. The ratio of actinomycetes to bacteria shows even more definite relationships to the temperature and moisture, as shown in Table 2. It is seen here that in every case, except No. IV, 25·5% H_2O, an increase in the temperature results in a narrowing of the ratio. Increased moisture has in most cases widened the ratio, or else failed to have any pronounced effect, such as in Exp. VI and X at room temperature.

TABLE 1.

Influence of Organic Materials on the Composition of the Soil Microflora.

Soil and Addition.	Temperature. °C.	H_2O. %.	Incubation. Days.	Bacteria.*	Actinomycetes.*	Fungi.		
						Plate Count.†	Density of Mycelium. %.	Spores. Average per 100 Microscopic Fields.
I. Heavy loam, rich in organic matter, pH 5·5, plus 2% $CaCO_3$ and 1·0% saccharose.	18–20	22·5	5	467·1	669·7	4,387	(rich)	(many)
		30·5	,,	2,402·6	374·1	—	(rich)	(many)
		35·5	,,	1,852·9	124·0	—	(rich)	(few)
	37	22·5	5	141·9	709·7	203	(scant)	(few)
		30·5	,,	172·7	141·9	—	(scant)	(few)
		35·5	,,	163·5	407·0	—	(scant)	(none)
II. Heavy loam, rich in organic matter, plus 4% $CaCO_3$ and 1·0% soluble starch.	16–18	27·5	4	156·9	29·8	71	1·1	3
			10	327·6	172·4	101	2·5	1
	28	27·5	4	424·1	346·9	84	4·8	1
			10	310·3	268·9	174	11·1	2
	41	27·5	4	93·1	153·8	30	1·3	1
			10	113·8	300·0	76	0·4	1

* Millions per gram of dry soil.

† Thousands per gram of dry soil.

TABLE 1.—*Continued.*

Influence of Organic Materials on the Composition of the Soil Microflora.—Continued.

Soil and Addition.	Temperature. °C.	H_2O. %.	Incubation. Days.	Bacteria.*	Actinomycetes.*	Fungi.		
						Plate Count.†	Density of Mycelium. %.	Spores. Average per 100 Microscopic Fields.
III. Loam, rich in organic matter, pH 7·3, plus 0·75% xylan.	18–21	13·0	6	1,139·4	54·6	402	42·5	2
		22·5	,,	1,858·0	17·7	245	31·0	1
	39	13·0	6	563·2	327·6	4,885	29·6	136
		22·5	,,	398·4	64·5	4,549	20·5	159
IV. Same as III, plus 1·0% hay meal (mixture of young grass and clover).	18–21	15·0	6	647·0	270·6	676	54·6	5
		19·5	,,	764·0	233·0	590	(rich)	(none)
		25·5	,,	596·0	90·6	537	(rich)	(none)
	39	15·0	6	216·2	133·8	8,235	24·4	172
		19·5	,,	(Lost)		10,311	(rich)	(many)
		25·5	,,	532·5	82·8	1,644	(rich)	(many)
V. Sand, very poor in organic matter, pH 5·1, plus 1·0% hay meal.	18–21	4·9	6	562·6	2·6	571	58·3	18
			14	429·8	21·0	570	48·4	13
		10·3	6	376·2	(0)	452	44·7	11
			14	360·9	5·6	309	24·0	11
	40	4·9	6	195·3	17·1	71	16·9	4
			14	141·6	34·6	166	1·3	14
		10·3	6	178·4	11·1	56	22·0	1
			14	118·5	29·4	27	4·2	1
VI. Sand, same as V, plus 1·0% $CaCO_3$ and 1·0% hay meal.	18–21	5·5	5	1,304·2	1·3	222	48·5	1
			12	571·4	1·1	180	36·0	10
		10·8	5	731·5	2·8	242	29·4	4
			12	338·0	2·2	153	29·8	7
	40	5·5	5	316·1	43·6	77	11·2	6
			12	105·8	25·4	28	1·4	3
		10·8	5	316·7	14·0	31	2·4	4
			12	111·4	16·4	5	1·9	0
VII. Same as V, plus 0·5% dried mycelium of actinomycetes.	18–20	4·8	5	625·0	(0)	158	(rich)	(few)
		9·4	,,	524·3	(0)	221	(rich)	(few)
	39	4·8	5	252·1	94·5	1,815	(scant)	(many)
		9·4	,,	275·9	33·1	154	(scant)	(few)

* Millions per gram of dry soil.

† Thousands per gram of dry soil.

TABLE 1.—*Continued.*

Influence of Organic Materials on the Composition of the Soil Microflora.—Continued.

Soil and Addition.	Temperature. °C.	H_2O. %.	Incubation. Days.	Bacteria.*	Actinomycetes.*	Fungi.		
						Plate Count.†	Density of Mycelium. %.	Spores. Average per 100 Microscopic Fields.
VIII. Red loam, pH 6·0, plus 1·0% hay meal.	18–21	17·0	5	551·7	123·5	133	31·6	0
	39	17·0	5	236·5	158·1	3,705	21·4	158
IX. Red loam, pH 6·8, plus 1·0% hay meal.	17–19	17·5	6	401·2	18·2	109	41·1	1
	28	17·5	6	175·8	130·9	945	21·0	18
	39	17·5	6	101·8	146·7	315	14·0	26
X. Heavy loam, rich in organic matter, pH 5·5, plus 1·0% hay meal.	16–19	18·2	5	442·4	1·5	206	46·3	1
			12	363·7	17·7	1,956	67·1	44
		25·0	5	756·7	4·2	300	44·6	1
			12	580·0	28·0	833	57·5	19
		30·0	5	912·5	4·5	300	55·6	0
			12	298·2	14·3	446	53·5	8
	27	18·2	5	287·3	35·1	3,545	57·3	82
			12	303·2	70·9	4,303	34·6	58
		25·0	5	540·0	48·3	2,233	47·5	43
			12	409·3	74·7	2,960	20·5	53
		30·0	5	310·7	57·1	1,821	55·8	21
			12	162·9	82·1	1,600	14·5	32
	39	18·2	5	68·9	35·1	7,182	49·6	147
			12	52·0	38·5	7,319	13·8	72
		25·0	5	155·0	44·4	3,700	46·4	135
			12	57·7	40·3	4,933	11·3	78
		30·0	5	80·4	39·3	3,357	31·7	36
			12	52·5	31·1	2,943	15·5	43

* Millions per gram of dry soil.

† Thousands per gram of dry soil.

We see thus, that with increasing temperature and decreasing moisture the balance of the microflora is shifted more and more from the bacteria towards the actinomycetes. This general principle, which is also supported by the appearance of the microscopic slides, and which applies both to different soils and different kinds of organic matter, is in full agreement with results previously obtained on soil samples taken from the field (Jensen, 1934*a*), but in the present experi-

ments, where extra organic matter is added, the influence of the temperature appears more important than that of the moisture, in contrast to the previous experiments. It is here to be noted that the limits of temperature and moisture were not precisely the same in the two series of experiments.

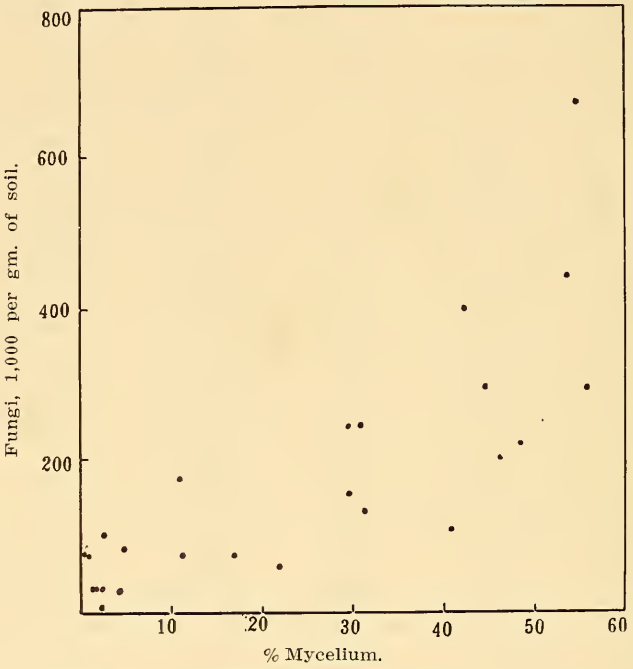
TABLE 2.

Ratios of Actinomycetes to Bacteria in Relation to Temperature and Moisture.

Soil No.	H_2O%.	Incubation, Days.	Ratio of Actinomycetes to Bacteria at:		
			Room Temperature.	27–28° C.	37–41° C.
I	22·5	5	1·43	—	5·00
	30·5	,,	0·156	—	0·96
	35·5	,,	0·067	—	2·50
II	27·5	4	0·231	0·854	1·65
	,,	10	0·526	0·709	2·64
III	13·0	6	0·048	—	0·582
	22·5	,,	0·010	—	0·162
IV	15·0	6	0·418	—	0·619
	19·5	,,	0·305	—	—
	25·5	,,	0·153	—	0·155
V	4·9	6	0·005	—	0·087
	10·3	,,	(0)	—	0·062
	4·9	14	0·049	—	0·259
	10·3	,,	0·015	—	0·062
VI	5·5	5	0·001	—	0·138
	10·8	,,	0·004	—	0·044
	5·5	12	0·002	—	0·239
	10·8	,,	0·006	—	0·147
VII	4·8	5	(0)	—	0·375
	9·4	,,	(0)	—	0·120
VIII	17·0	5	0·224	—	0·669
IX	17·5	6	0·044	0·745	1·44
X	18·2	5	0·003	0·122	0·511
	25·0	,,	0·006	0·089	0·215
	30·0	,,	0·005	0·184	0·489
	18·2	12	0·049	0·234	0·741
	25·0	,,	0·048	0·182	0·699
	30·0	,,	0·048	0·504	0·592

Table 1 also shows that the density of fungal mycelium (expressed as the percentage of microscopic fields showing presence of hyphae) is in 18 cases out of 21 higher, and often very much so, at 16–21° than at 37–41° C., regardless of the nature of the soil or the organic material added. At 27–28° C. the density is in 4 cases out of 9 lower than at room temperature, but in 7 cases higher than at 37–41° C. Experiments V, VI and X show that the strongest development of

mycelium takes place within the first 4–6 days, after which time the figures for the density of mycelium drop markedly at higher temperatures, while often remaining quite high or even increasing at room temperature. It is also noteworthy that increased moisture generally tends to reduce the development of mycelium, and that the alkaline soils No. IV and VI are with equal treatment nearly as rich in mycelium as the acid soils No. V and X. The plate counts of fungi tell quite a different story. They show in Experiments III, IV, VII and X a huge increase in fungi at 27–28°, and especially 37–41° C. At the last



Text-figure 1.—Correlation between density of mycelium and plate counts of fungi in 25 cases from Table 1.

temperatures the fungous flora was remarkably uniform in composition, mainly consisting of a green *Aspergillus*, probably *fumigatus*, and a species of *Monilia*, whereas Mucoraceae, Penicillia and Fusaria predominated at the other temperatures. The last column of Table 1 shows that in all cases of high plate-counts there is also a very large number of fungal spores present on the slides; the conidiophores of the *Aspergillus* and the spore-chains of the *Monilia* were clearly recognizable on such slides. Although, as stated above, these figures may not be reliable indices of the actual density of fungal spores, they nevertheless show a fine correlation with the plate counts; the correlation coefficient amounts to 0·865, a value which remains practically unaltered (0·864) when calculated as a partial correlation coefficient with constant density of mycelium. On the other hand, the figures for density of mycelium do not, when the whole set of data is considered, show any correlation with the plate counts. The correlation coefficient between these two values is only 0·112, and this value even disappears (0·036) when

calculated as a partial correlation coefficient with constant number of spores. This shows conclusively that the plate counting under these conditions measures only the number of fungal spores in the soil (cf. McLennan, 1928, and Conn, 1932). However, a real correlation between density of mycelium and plate counts appears if we consider only those 25 cases in Table 1, where the microscopical examination has shown an average of less than 10 spores per 100 fields (Text-fig. 1). Here the correlation coefficient between density of mycelium and plate counts amounts to 0·769, a value of very high significance with 25 observations (Fisher, 1930, Table V. A). The correlation coefficient between spore-numbers and plate counts is here insignificant (0·301). This shows us that the plate count is an index of the density of fungal mycelium only in cases where the number of spores is low in proportion to the amount of vegetative mycelium, but as the numbers of spores increase, they influence the numbers of colonies so strongly that the amount of vegetative mycelium becomes relatively unimportant.

A calculation of the distribution of χ^2 (Fisher, 1930) from the 61 counts of fungi* in Table 1 and the 9 in Table 3 gave the following result:

Value of χ^2.	Frequency.	
	Expected.	Observed.
0·0	0·7	0
0·115	0·7	1
0·185	2·1	0
0·352	3·5	3
0·584	7	9
1·005	7	14
1·424	14	12
2·366	14	17
3·665	7	5
4·642	7	6
6·251	3·5	1
7·815	2·1	1
9·873	0·7	0
11·341	0·7	1

The agreement with expectation is here reasonably good, and the difference $\sqrt{2\chi^2} - \sqrt{2n-1}$ was found to be $-1{\cdot}11$. We may, therefore, conclude that the plate method is satisfactory for the determination of the number of fungus spores capable of growing on agar. But it is evident that, if we want to estimate the importance of fungi versus other microorganisms in biochemical soil processes, the plate counts may not be of much help, since the metabolic processes will presumably be carried out by the vegetative mycelia and not by resting spores which may be present in vast numbers in soil to which organic matter has been added. Here the determination of the density of mycelium by the microscopic method promises to be of value, particularly since this method has, in comparison with McLennan's (1928) method for distinguishing between spores and mycelium, the advantage of showing the presence of mycelia incapable of growth in artificial media although possibly capable of producing biochemical changes. A tentative

* Four parallel plates in each set.

experiment may be quoted: a "synthetic soil" was made up from 80% pure sand, 18·5% pure kaolin, 1% calcium carbonate, and 0·5% ferric oxide; to this mixture were added 1% hay meal and 11·5% water. Three series of experiments were run: at room temperature (16–19° C.), at 28° C., and at 38° C. The production of carbon dioxide was determined daily,* and plate counts of bacteria, actinomycetes and fungi as well as estimations of the density of fungal mycelium on microscopic slides were carried out after 4, 8 and 14 days. The results, shown in Text-figure 2 and Table 3, agree well with those previously obtained. The numbers of bacteria and actinomycetes are higher at room temperature, and particularly at 28° C. than at 38° C., and the ratio actinomycetes:bacteria becomes narrower with increasing temperature (Table 3). The development of mycelium, which reaches its maximum by the fourth day, approximately coinciding with a maximum in the production of carbon dioxide, remains considerable at room temperature, but decreases rapidly at the two higher temperatures. While the numbers of bacteria plus actinomycetes and the densities of mycelium show a fairly definite correlation with the carbon dioxide formation, the plate counts of fungi (Table 3) merely show figures increasing steeply with both the time and the temperature, due to production of spores (particularly of *Asp. fumigatus* at 38° C.) and entirely uncorrelated with the rate of carbon dioxide formation. It is noteworthy that the increased carbon dioxide production at 38° C. is not brought about by an increased number of microorganisms; other experiments with direct microscopic counting of the bacteria, to be described later, have given similar results.

TABLE 3.

Numbers of Fungi and Ratios of Actinomycetes to Bacteria in Sand-Kaolin-Mixture plus Hay Meal.

Incubation Period.	16–19° C.		28° C.		38° C.	
	Fungi.*	Ratio A : B.	Fungi.*	Ratio A : B.	Fungi.*	Ratio A : B.
4 days	474	0·0041	2,281	0·0394	5,010	0·147
8 „	1,339	0·0252	3,692	0·0734	38,670	0·249
14 „	3,213	0·092	6,313	0·0973	44,420	0·262

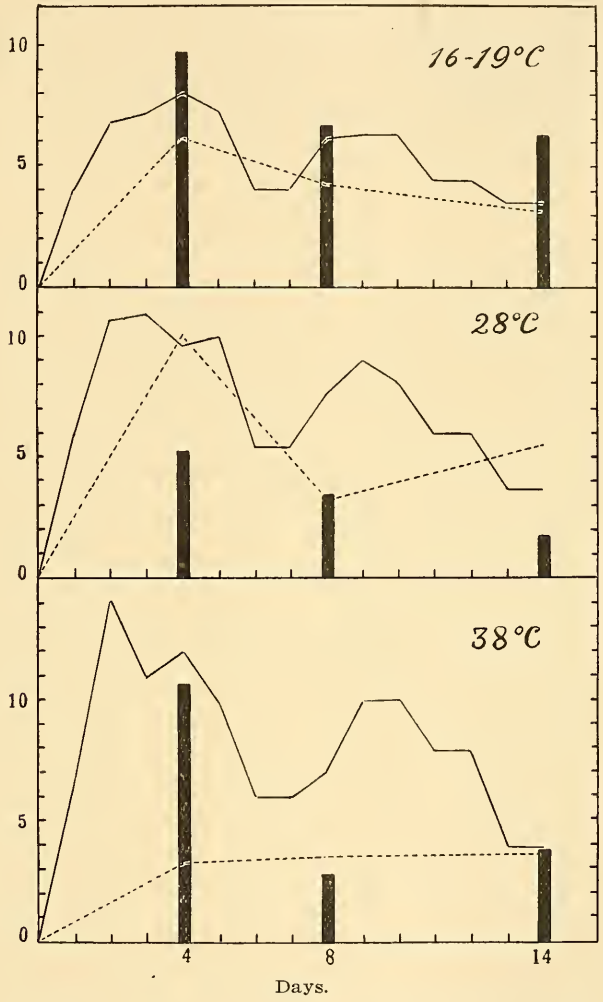
* Thousands per gram of dry "soil".

Summary.

The influence of different kinds of organic material, mostly hay meal, on the microorganisms of various soils was studied at temperatures from about 16° to about 40° C. and at varying degrees of moisture. The multiplication of bacteria, as determined by plate counting, was found generally to be strongest at the lower temperatures, but the reverse applied to the actinomycetes. The ratio of actinomycetes was narrowest at high temperature and low moisture. A quantitative estimate of the vegetative development of fungi was obtained by determining the percentage of microscopic fields showing the presence of fungal hyphae on microscopic slides placed in the soil, according to the method of Rossi and Cholodny. By this method it was found that the fungi generally

* The arrangement of these experiments and the method of carbon dioxide determination will be described in detail in a subsequent paper.

attain their most abundant vegetative development at 16–21° C., and that high plate counts of fungi at higher temperatures are merely due to an abundant production of spores. The method of plate counting, as applied to fungi, seems satisfactory only for the determination of the number of fungal spores in the soil. The figures for the density of mycelium, obtained by the microscopic



Text-figure 2.—Correlation between carbon dioxide production, density of fungal mycelium and numbers of bacteria plus actinomycetes in sand-kaolin-mixture plus hay meal.—Unbroken line: production of CO_2 in 24 hours; unit of scale: 10 mgm. CO_2 per 100 gm. dry material.—Dotted line: numbers of bacteria plus actinomycetes; unit of scale: 40 millions per gm. of dry material.—Black columns: density of fungal mycelium; unit of scale: 5 per cent. fields showing presence of hyphae.

method, as well as the plate-counted numbers of bacteria and actinomycetes, showed a correlation with the rate of carbon dioxide production.

References.

CONN, H. J., 1932.—The Cholodny Technic for the Microscopic Study of the Soil Microflora. *Cent. f. Bakt.*, ii, 87, 233-239.

FISHER, R. A., 1930.—Statistical Methods for Research Workers. (Third Ed., London and Edinburgh.)

JENSEN, H. L., 1934*a*.—Contributions to the Microbiology of Australian Soils. i. PROC. LINN. SOC. N.S.W., lix, 101-117.

————, 1934*b*.—Contributions to the Microbiology of Australian Soils. ii. PROC. LINN. SOC. N.S.W., lix, 200-211.

MCLENNAN, E., 1928.—The Growth of Fungi in Soil. *Ann. Appl. Biol.*, 15, 95-109.